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Award Number: DAMD17-00-1-0229

TITLE: Functional Role of the Casein Kinase I (CKI) Family
in the Transforming Growth Factor- β (TGF- β) Signaling
Pathway

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REPORT DATE: April 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20040329 045

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE April 2003		3. REPORT TYPE AND DATES COVERED Annual Summary (1 Apr 00-31 Mar 03)
4. TITLE AND SUBTITLE Functional Role of the Casein Kinase I (CKI) Family in the Transforming Growth Factor- β (TGF- β) Signaling Pathway			5. FUNDING NUMBERS DAMD17-00-1-0229	
6. AUTHOR(S) David Waddell Nicole T. Liberati, Ph.D. Xiao-Fan Wang, Ph.D.				
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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The regulation of the Transforming Growth Factor- β (TGF- β) signaling pathway and its role in cancer is an area of intense research. We are investigating the regulatory role of casein kinase I (CKI) in the TGF- β signaling cascade. We have found that all CKI isoforms (α , δ , ϵ , and γ) can bind to Smads <i>in vitro</i> and that this interaction is mediated through the Mad Homology 2 (MH2) domain of the smad proteins. Furthermore, we have shown that CKI ϵ can interact with smads and receptors <i>in vivo</i> , and that the CKI ϵ /Type I and CKI ϵ /Type II receptor interactions is independent of TGF- β ligand activity, while CKI ϵ /Smad interaction is transiently disrupted by ligand stimulation. Since, CKI family members are serine/threonine kinase, we determined that CKI ϵ and CKI γ 2 can both phosphorylate the receptor-activated Smads and the Type II Receptor, while CKI γ 2 can also phosphorylate the co-smad, Smad4 <i>in vitro</i> . Transcriptional reporter assays revealed that in the absence of TGF- β , transient overexpression of CKI ϵ or CKI γ 2 dramatically reduced basal reporter activity, but in the presence of ligand CKI ϵ enhanced and CKI γ 2 inhibited TGF- β mediated transcription. Furthermore, CKI ϵ is also capable of significantly enhancing the transcriptional activity of smad3. Finally, we have shown that transient siRNA knock-down of all CKI isoforms resulted in dramatic increases in both basal and ligand stimulated transcriptional reporter activity. Taken together, these observations provide exciting evidence for a functional role for CKI in the TGF- β pathway, a pathway that has been shown to be involved in the development and progression of many different types of cancers.				
14. SUBJECT TERMS Transforming Growth Factor- β (TGF- β), Casein Kinase I ϵ (CKI ϵ), Casein Kinase I γ 2 (CKI γ 2)				15. NUMBER OF PAGES 49
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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Introduction

The Transforming Growth Factor- β (TGF- β) signaling pathway has been shown to be critical to the processes of embryological development of organisms as diverse as fruit flies and humans. This pathway can be detected at very early stages of development and acts to coordinate the complex mechanisms of cellular differentiation that will ultimately result in a mature organism. The TGF- β pathway continues its regulation of cellular events during the developmental stages and throughout the lifespan of more complex organisms. The power that this signaling cascade has over cellular fate is necessary for its ability to regulate development and differentiation, however when regulatory controls are lost, the result is usually uncontrolled growth and proliferation. Therefore, it is not surprising that mutations within the TGF- β pathway have been implicated in a wide range of clinically observed oncogenic lesions including breast cancer.

The TGF- β superfamily of ligands includes the bone morphogenetic proteins (BMPs), activin and TGF- β . The signaling pathway is a relatively simple cascade that consists of the ligand, the type I and type II receptors, and the cytoplasmic signal transducers called smads (for a more detailed review of this pathway refer to references 54-56) . The type I and type II receptors are serine/threonine kinases that, upon ligand binding, form a heterotetrameric complex in which the constitutively active type II receptor phosphorylates the type I receptor in the GS domain resulting in catalytic activation. The activated type I receptor then transiently associates with and phosphorylates the receptor activated smads (R-smads) at their two most C-terminal serine residues. The smad proteins consist of two highly conserved mad homology domains, termed MH1 and MH2, connected by a relatively divergent linker region. The MH1 domain is involved

in DNA binding, while the MH2 domain is important for protein/protein interactions. The mad homology domains are capable of interacting with each other in an inhibitory fashion that is alleviated by type I receptor phosphorylation. This phosphorylation results in association with the co-smad, translocation to the nucleus, and regulation of gene transcription usually through association with coactivators, corepressors, or other transcription factors such as AP-1 or the Wnt regulated Lef/Tcf family members.

The casein kinase I (CKI) family has seven identified isoforms (α , β , $\gamma 1$, $\gamma 2$, $\gamma 3$, δ , and ϵ) that possess a highly homologous N-terminal kinase domain and a highly divergent C-terminal tail, and have a predicted molecular weight of approximately 40-50kDa (for a detailed view of the CKI family refer to reference 1). These kinases have been implicated in a wide range of cellular functions including, vesicular trafficking, DNA damage repair, cell cycle progression, and cytokinesis. CKI was one of the first serine/threonine kinases ever purified and hence extensive research has been done on characterizing its activity, substrate specificity, function, tissue distribution, subcellular localization and regulation. The results of this research have lead to the characterization of the general consensus phosphorylation sequence S/T/Y(P)X₁₋₃S/T (40,41). This sequence suggests that the action of other kinases is probably required for CKI activity, and thus CKI has been classified as a phosphate-directed kinase. Since it is not clear how these constitutively active kinases are regulated within a cell, it appears that this may be one major mechanism by which control is achieved, with subcellular localization being a second likely mechanism. The CKI family also possesses two other potentially interesting physical features, the first being a kinesin homology domain (KHD) and the second being a near consensus SV40 T antigen nuclear localization sequence (NLS) (1). The significance of these

sequences remains to be determined however, a recent paper has demonstrated that the NLS sequence is definitely functional and necessary for nuclear translocation of CKI α (27). The majority of the research done on the CKI family has focused primarily on characterizing their function and identifying potential substrates. However, over the last several years there has been a revolution in the CKI field and this obscure family of kinases has moved into a position of intense research in the field of signal transduction. The last several years have seen the publication of numerous papers that demonstrate a significant role for CKI ϵ and CKI δ in the circadian rhythms of mammals (17-19), the cytoplasmic sequestration of NFAT and the regulation of a G $_{q/11}$ -coupled receptor by CKI α (25,26), the regulation of the β -PDGFR by CKI γ 2 (23), and the positive regulation of the Wnt signaling pathway by CKI ϵ (9-12). These findings combined with our own preliminary results has resulted in our undertaking the task of determining if the CKI family plays a functional role in the TGF- β signaling pathway and what the significance of this role may mean with respect to the development and progression of breast cancer.

Body/Results

Task 1:

Determine if CKI family members can physically interact with components of the TGF- β pathway and whether these interactions are ligand dependent.

Several years ago a yeast two-hybrid screen was performed in the lab using smad3 as bait. This screen generated several hundred clones, each representing a potential smad3 interactor and TGF- β pathway effector. The results of this screen have since acted as a launching pad for further investigation into identifying and characterizing these proteins and elucidating their potential role within the TGF- β pathway. One protein that has been identified on at least three separate occasions is Casein Kinase I γ 2 (CKI γ 2). The identification of this kinase as a potential interactor was interesting in and of itself, but with the publication of a study implicating CKI ϵ (a closely related homolog of CKI γ 2) in the regulation of the Wnt pathway (9-12), our interest became more focused and we began determining if the CKI family might play some role in regulating the TGF- β signaling cascade.

CKI ϵ interacts strongly with Smads *in vitro*, while CKI α / δ / γ 2 family members interact weakly.

In order to determine which CKI isoforms are present in the TGF- β responsive cell lines we routinely use in the laboratory (namely HepG2 and HaCaT cells), we performed RT-PCR using specific primers for each of the seven known isoforms. As shown in Figure 1, CKI α / γ 2/ δ / ϵ are expressed on both HepG2 and HaCaT cells, but CKI γ 1 and CKI γ 3 do not show any detectable expression (the gel shown in Figure 1 is representative of the results obtained for both HepG2 and HaCaT cell lines individually). Based on this data, we next performed GST pulldown assays for each CKI family member that was shown to be expressed in our TGF- β responsive cell lines.

CKI family members were radiolabeled *in vitro* with Methionine-S³⁵ and GST pulldown assays were performed using purified GST fusions of smad1, smad2, smad3, smad4, and smad5. As shown in Figure 2, CKIε is able to bind strongly to smad1/2/3/4, but weakly to smad5, while the CKIα and CKIδ interactions with the smad proteins is relatively much weaker *in vitro*. Furthermore, using constructs in which GST is fused to the MH1 domain (S3N), the MH1 domain and linker region (S3NL), the MH2 domain (S3C) or the MH2 domain and linker region (S3CL) of smad3, it appears that CKIα, CKIδ, and CKIε all have the most high affinity for the MH2 domain (Fig. 1). This point may prove important as a functional role for the CKI family in the TGF-β pathway is developed.

The CKI family members each contain relatively divergent tail regions that extend beyond the highly conserved kinase domain, with the tail region of CKIε being relatively long. It has been suggested that these tail regions may play an important role in substrate binding and ultimately providing a mechanism by which each isoform maintains some level of specificity. Therefore, with this idea in mind we introduced premature stop codons by site directed mutagenesis into the tail region of CKIε in order to produce C-terminal truncations. These mutants were then fused to GST, purified and used in *in vitro* binding assays with radiolabeled full length smads. As shown in Figure 5, the tail region of CKIε is not necessary for the interaction with smad proteins, thus suggesting that the kinase domain must also participate in the binding of smads.

Initial *in vitro* binding studies using radiolabeled full length CKIγ2 resulted in virtually undetectable binding to any of the GST purified smads. This finding was especially surprising, since this was the isoform that was originally identified in the yeast two-hybrid screen in which smad3 was used as bait. Further analysis of the CKIγ2 sequences that were isolated in the yeast

two-hybrid assay showed that none contained the N-terminal portion of the protein. Therefore, we fused CKI γ 2 lacking the N-terminus to GST and performed a GST pulldown assay using smads radiolabeled with Methionine-S³⁵. As shown in Figure 3, the elimination of the N-terminus of CKI γ 2 resulted in strong binding to smad1, detectable binding to smads3/4, but no detectable binding to smad2. This data suggests that the unique N-terminal extension preceding the kinase domain of CKI γ 2 must somehow inhibit *in vitro* binding with smads. Furthermore, GST pulldown assays were performed in which radiolabeled full-length smad proteins or radiolabeled smad proteins with approximately the last 30 amino acids deleted from the C-terminus (smad- Δ C) were incubated with GST-CKI γ 2- Δ N bound to glutathione-sepharose beads. The results of these pulldown experiments showed a complete loss of binding between CKI γ 2 and the smad- Δ C proteins (Figure 4 shows the interaction between CKI γ 2- Δ N and Smad1 or Smad1- Δ C and is representative of what was observed with the other smad proteins that were evaluated). This data provided strong evidence that the last 30 amino acid residues of the smad proteins are necessary for interaction with CKI γ 2.

Full length CKI ϵ can interact with the TGF- β Type I and Type II Receptors *in vitro*.

It was previously observed that immunoprecipitated TGF- β type II receptors possessed, what was believed at the time to be, an intrinsic casein kinase I activity (39). Close examination of the data shows a coimmunoprecipitated protein of about 40-45kDa, the approximate size of CKI. This observation lead us to hypothesize that CKI ϵ may be capable of interacting with the TGF- β type I and type II receptors. Therefore, CKI ϵ was radiolabeled *in vitro* with Methionine-S³⁵ and GST pulldown assays were performed with purified GST fusions of the cytoplasmic domains of the TGF- β type I and type II receptors, as well as, GST fusions of the TGF- β type II receptor in

which various regions have been deleted. As shown in Figure 6, CKI ϵ is capable of binding to the type I and the type II receptors, as well as all of the type II receptor deletion constructs.

CKI ϵ can interact with Smads and TGF- β Type I and Type II Receptors *in vivo*.

Since CKI ϵ can bind to smads and receptors *in vitro*, we next decided to look whether this interaction also occurred in living cells. HaCaT-CKI ϵ 3 cells, a spontaneously immortalized human keratinocyte cell line that is responsive to TGF- β ligand and stably overexpresses CKI ϵ , were used for these co-immunoprecipitation assays. Cell lysates were incubated with one of the following; anti-TGF- β type I, anti-TGF- β type II, anti-smad 2/3 or anti-smad 1/5, and then blotted for CKI ϵ . As shown in Figure 7, CKI ϵ can interact with receptors and smads *in vivo*. Furthermore, it appears that the strongest interaction occurs with the receptors, while a weaker interaction is seen with the receptor activated smads. We have also done a co-immunoprecipitation assay with anti-smad4 and found that CKI ϵ and smad4, the co-smad, can interact *in vivo*. Although parental HaCaT cells express CKI ϵ endogenously and association with smads and receptors can be observed when co-IP experiments are done using the wild type cells, we decided to use the HaCaT-CKI ϵ 3 cells for these studies so that the interactions would be easier to detect and monitor. This point becomes most important when co-IP experiments are done with the smad antibodies due to the relatively weak signal observed with the endogenous proteins alone (Figure 7).

TGF- β treatment transiently disrupts the CKI ϵ /Smad interaction, but does not effect the CKI ϵ /Receptor interaction.

The *in vivo* interaction with components of the TGF- β pathway provided some evidence that there may be a functional role played by CKI ϵ . The next question we wanted to address was

whether treatment of the stably transfected HaCaT cells with TGF- β ligand might affect these interactions. Cells were treated over a four hour period with TGF- β ligand, the cells were lysed, lysates were incubated with anti-smad2/3, and blots were performed for CKI ϵ . As shown in Figure 8, the interaction between CKI ϵ and Smad2/3 is transiently disrupted with TGF- β treatment. However when the same experiment was performed for the TGF- β type II receptor, there was no observable disruption of CKI ϵ binding (Figure 9). We have also performed this experiment probing for the TGF- β type I receptor and again found that there is no observable disruption of CKI ϵ binding (data not shown).

Task 2:

Examine whether CKI family members can regulate TGF- β mediated gene transcription.

In order to determine if CKI isoforms are capable of playing a functional role in the TGF- β pathway, we decided to use the classical transcription reporter assay as a measure of function. There are several reporter constructs that are widely used to monitor TGF- β regulated transcription. We tried two different constructs, the first is the 3TP-Lux construct that consists of a region of the PAI-1 promoter that is known to contain smad binding elements as well as AP-1 binding elements (47). The second is a concatemericized smad binding element (SBE) fused to the luciferase gene, with no AP-1 sites present. HepG2 cells, a human hepatocellular carcinoma cell line, are responsive to TGF- β and easily transfectable. Using the SBE reporter construct, we found that transient overexpression of CKI ϵ resulted in reduced basal transcriptional activity, while at the same time this overexpression actually enhanced TGF- β stimulated transcriptional activity. This resulted in a dramatic increase in fold TGF- β induction with the addition of CKI ϵ compared to control. Furthermore, a kinase dead construct of CKI ϵ (CKI ϵ -KD) was also capable

of reducing basal transcriptional activity, but failed to enhance activity in response to TGF- β (Figure 12 and data not shown). These results suggest that the reduction in basal activity only requires the presence of the protein and not the kinase activity, while enhancement of TGF- β ligand treatment requires the protein and the kinase activity. In addition, using the 3TP-Lux reporter, HepG2 cells show a significant increase in transcriptional activity in response to TGF- β which is further increased by the overexpression of smad3, however when smad3 and CKI ϵ are added together the response to TGF- β is increased by approximately twice that seen with TGF- β and smad3 alone (Figure 13). Furthermore, when Smad2 and CKI ϵ were added together there was no enhancement over that seen for smad2 alone (Figure 13), which is expected since smad2 requires smad4 for binding DNA. The enhancement of smad3 transcriptional activity was also observed using the SBE-Lux reporter (Figure 14). These results strongly suggest that CKI ϵ is capable of enhancing the transcriptional activity of smad3 by some unknown mechanism.

In contrast to the apparent dual functional role of CKI ϵ within the TGF- β signaling pathway, CKI γ 2 appears to be a strong negative regulator of this cascade. As shown in Figure 15, when HepG2 cells are transiently transfected with CKI γ 2 the response of the 3TP-Lux reporter to TGF- β is almost completely negated. Furthermore, Figure 15 also shows that CKI γ 2 reduces the transcriptional activity of smad3 by nearly 50% when both are overexpressed simultaneously. This result is in direct contrast with the results obtained for the CKI ϵ and smad3 overexpression reporter assays described above (Figure 13 and Figure 14). The functional differences observed between CKI ϵ and CKI γ 2 in the TGF- β transcriptional reporter assays are further supported by the data that is described in the following sections. Based on the functional transcriptional reporter assays conducted to date, our data demonstrates that different CKI isoforms appear to

perform distinct and sometimes opposite functions within the TGF- β signaling pathway. These observations are extremely interesting and, combined with previously published data, suggest that the CKI family members may play important functional roles within a cell by providing a second level of regulatory control of many different signaling cascades.

Task 3:

Determine if CKI family members are necessary to maintain normal TGF- β pathway function.

The seemingly opposing effects on the TGF- β signaling pathway that resulted from overexpressing CKI ϵ and CKI γ 2 provided us with an insight into the potential functions of these two kinases. In order to further prove that these kinases actually did play an important regulatory role, we decided to transiently knockdown the expression of individual CKI isoforms in HepG2 cells and then perform transcriptional reporter assays in order to determine if there was any change in basal or ligand stimulated reporter activity. We designed siRNA oligos directed against, CKI ϵ alone, CKI α alone, CKI γ 2 alone, or CKI δ/ϵ together. The rationale for designing a siRNA oligo against CKI ϵ and CKI δ simultaneously was because it has been previously shown that these two isoforms have very similar amino acid sequences and have overlapping functions within a cell. Therefore, in order to determine if the loss of CKI ϵ would result in any change in the TGF- β pathway we felt reducing CKI δ might also be necessary. As shown in Figure 16, this hypothesis proved to be accurate and is illustrated by the data showing that the siRNA against CKI ϵ alone only resulted in a small increase in both basal and ligand stimulated reporter activity. In contrast, when the siRNA directed against both CKI ϵ and CKI δ was used, there was a dramatic increase in both basal and ligand stimulated report activity. Furthermore, transient knockdown of CKI α also resulted in a large increase in both basal and TGF- β stimulated reporter

activity suggesting that these three isoforms all have at least some overlapping functions. Finally, the knockdown of CKI α also resulted in a complete loss of CKI ϵ expression suggesting that some CKI isoforms may also be responsible for regulating the expression of other CKI isoforms (Figure 16).

The overexpression and transient knockdown experiments employed to elucidate the functional roles of CKI α , CKI ϵ , and CKI δ , within the TGF- β signaling pathway, have unveiled a group of CKI isoforms that are capable of carrying out similar and overlapping functions within the cell. However in contrast to CKI $\alpha/\delta/\epsilon$ which appear to have a dual functional role in regulating this pathway, CKI γ 2 is undeniably a negative regulator of TGF- β signaling. The initial transcriptional reporter assays in which CKI γ 2 overexpression was analyzed for effects on TGF- β signaling provided us with our first evidence that this isoform was a potent inhibitor of this cascade. We next decided to compliment the overexpression data by transiently knocking-down CKI γ 2 expression and then monitoring for any changes in TGF- β simulated transcriptional activity. We designed three siRNA sequences against CKI γ 2 and determined the effectiveness of each construct by western blot analysis using a rabbit polyclonal antibody we raised against the C-terminus of CKI γ 2. As shown in Figure 18, all three sequences dramatically reduced protein levels. Furthermore, the reduction of CKI γ 2 protein levels resulted in a significant increase in ligand stimulated reporter activity (Figure 17), which is consistent with the inhibitory effects observed when this isoform was overexpressed (Figure 15).

Task 4:

Determine if CKI family members can phosphorylate components of the TGF- β pathway, identify potential phosphorylation sites, and evaluate the functional significance of these sites.

Since the CKI isoforms are serine/threonine kinases (same as the TGF- β type I and type II receptors), we wanted to see if CKI ϵ and CKI γ 2 could phosphorylate purified smads *in vitro*. We were also curious to see if either kinase might be able to phosphorylate the type I and/or the type II receptors since we have observed that CKI ϵ is able interact with both receptors *in vitro* and *in vivo*. As shown in Figure 10, CKI ϵ phosphorylates the TGF- β activated smads (smads2/3) and the BMP activated smads (smads1/5) to a lesser extent, but it does not phosphorylate the co-smad (smad4). In addition, we observed that CKI ϵ appears to phosphorylate the MH1 domain and the linker region of smad3, but not the MH2 domain (the region phosphorylated by the type II receptor). Furthermore, CKI ϵ can phosphorylate the cytoplasmic region of the type II receptor, but does not appear to phosphorylate the type I receptor. The *in vitro* phosphorylation patterns of CKI γ 2 and CKI ϵ are very similar, however, as shown in Figure 11, CKI γ 2 not only phosphorylates all the receptor activated smads (smad1/2/3/5) but also the co-smad (smad4). Additionally, Figure 11 also shows that CKI γ 2 can phosphorylate smad3 in the MH1 domain, the linker region, as well as the MH2 domain. Finally we observed that CKI γ 2, similar to CKI ϵ , is only capable of phosphorylating the TGF- β type II receptor, but not the type I receptor.

To date, our attempts to identify CKI phosphorylation sites and evaluate their functional significance have proven difficult and laborious. The fact that there is no well defined CKI consensus phosphorylation sequence reduces the technique for identification of potential sites to random scanning mutagenesis of all serine and threonine residues within a defined region. We have begun the preliminary work of identifying potential sites in smad3, but have yet to conclusively identify any serines or threonines that show both a reduction in overall phosphorylation and a measurable change in functional assays when mutated to alanine.

Although this particular aim is labor intensive and time consuming, we will continue until we are satisfied that all potential sites have been properly analyzed.

Key Research Accomplishments

- Shown that many Casein Kinase I isoforms (CKI α , CKI γ , CKI δ , and CKI ϵ) are capable of interacting with multiple components of the TGF- β signaling pathway both *in vitro* and *in vivo*.
- Shown that Smad/CKI ϵ binding *in vitro* does not require the divergent C-terminal tail region of this kinase.
- Shown that the C-terminal region of Smads is required for CKI γ 2 binding *in vitro*.
- Shown that CKI ϵ binding to the receptor activated Smads *in vivo* is transiently disrupted by TGF- β ligand stimulation.
- Shown that CKI ϵ binding to the TGF- β type I and type II receptors is independent of TGF- β ligand stimulation.
- Shown that CKI ϵ acts to regulate TGF- β mediated transcription, as well as enhance the transcriptional activity Smad3.
- Shown that CKI γ 2 acts to inhibit TGF- β mediated transcription.
- Shown that CKI ϵ can phosphorylate the receptor activated smads and the cytoplasmic domain the TGF- β type II receptor *in vitro*.
- Mapped the CKI ϵ phosphorylation sites of Smad3 to the MH1 domain and the linker region.
- Shown that CKI γ 2 can phosphorylate all smads and the cytoplasmic domain the TGF- β type II receptor *in vitro*.
- Mapped the CKI γ 2 phosphorylation sites of Smad3 to the MH1 domain, the linker region, and the MH2 domain.
- Shown that transient overexpression of a kinase dead version of CKI ϵ acts as a dominant negative and inhibits TGF- β mediated transcription.
- Shown that transient knock-down of CKI family members using siRNA technology results in a dramatic increase in basal and ligand stimulated transcriptional reporter activity.

Reportable Outcomes

Manuscripts:

Casein Kinase I ϵ Plays a Functional Role in the Transforming Growth Factor- β Signaling Pathway. David S. Waddell, Nicole T. Liberati, Ph.D., and Xiao-Fan Wang. *Duke University Medical Center, Department of Pharmacology and Cancer Biology, Durham, NC 27710.* To be submitted to Journal of Biological Chemistry.

Casein Kinase I γ 2 Negatively Regulates the Transforming Growth Factor- β Signaling Pathway by Targeting Smad3 for Degradation. David S. Waddell, Guo Xing, Nicole T. Liberati, and Xiao-Fan Wang. . *Duke University Medical Center, Department of Pharmacology and Cancer Biology, Durham, NC 27710.* To be submitted to Journal of Biological Chemistry.

Abstracts:

Casein Kinase I ϵ Regulates the TGF- β Pathway and Provides a Link for TGF- β Activation of the Wnt Pathway. David S. Waddell, Nicole T. Liberati, Jeremy N. Rich, and Xiao-Fan Wang. *Duke University Medical Center, Department of Pharmacology and Cancer Biology, Durham, NC 27710.* Submitted for the April 2002 AACR Meeting.

Casein Kinase I ϵ Plays a Functional Role in the Transforming Growth Factor- β Signaling Pathway. David S. Waddell, Nicole T. Liberati, Ph.D., and Xiao-Fan Wang, Ph.D. *Duke University Medical Center, Department of Pharmacology and Cancer Biology, Durham, NC 27710.* Submitted for the September 2002 Era of Hope Meeting.

Degrees:

Mrs. Xuefang Bai, the original recipient of this award, was funded for one year prior to graduating with a Master of Science Degree in Molecular Cancer Biology.

David Waddell, the recipient of a transfer and revised statement of work for this award, was funded for the remaining two years and will graduate with a Ph.D. in Molecular Cancer Biology in the Spring of 2004.

Cell Lines and Serum:

HaCat cells, a spontaneously immortalized cell line that is responsive to TGF- β , has been stably transfected with CKI ϵ and CKI γ 2, as well as the kinase dead versions of these two proteins.

A polyclonal antibody has been raised in rabbits to the C-terminus of CKI γ 2.

Conclusions

The TGF- β signaling pathway has been shown to be involved in a wide range of biological processes, including development, differentiation and oncogenesis. The regulation of this pathway, and its role in cancer, continues to be an area of intense investigation. Recently Casein Kinase I (CKI) has been shown to regulate the Wnt pathway, another major pathway involved in the development of numerous types of cancers. In this study, we have engaged in an investigation to determine the regulatory role of CKI ϵ in the TGF- β pathway. This pathway consists of the ligand, the Type I and the Type II serine/threonine receptor kinases, which complex upon ligand binding, to activate a family of intracellular signal transducing proteins called Smads. We have found that CKI ϵ binds to all smads and the cytoplasmic domains of the Type I and Type II receptors both *in vitro* and *in vivo*. Furthermore, we have found that CKI α and CKI δ are also capable of binding to smads *in vitro*, but this interaction is much weaker than that observed for CKI ϵ . The interaction of CKI ϵ with the Type I and Type II receptors is independent of TGF- β ligand stimulation. However, the CKI ϵ /Smad interaction is transiently disrupted by TGF- β stimulation, with complete disassociation by 2 hours. Since CKI ϵ is also a serine/threonine kinase, we examined *in vitro* phosphorylation of Smads and receptors by CKI ϵ . Only the receptor-activated Smads (Smads 1, 2, 3, and 5) and the type II receptor are phosphorylated by CKI ϵ . Furthermore, using reporter assays it was observed that in the absence of TGF- β , CKI ϵ dramatically reduces basal transcriptional reporter activity, but in the presence of ligand CKI ϵ increases TGF- β mediated transcription. The enhancement of TGF- β mediated transcription is most likely the result of the ability of CKI ϵ to dramatically enhance Smad3 transcriptional

activity. Finally, by transiently reducing the protein levels of CKI α , CKI ϵ , or CKI δ and CKI ϵ together, it was found that these kinases are critically important for maintaining stable and controlled regulation of the TGF- β signaling pathway. The loss of these proteins inevitably resulted in dramatic increases in both basal and ligand stimulated transcriptional reporter activity in TGF- β responsive cells.

In addition to the results we have obtained for CKI $\alpha/\delta/\epsilon$, we have also found that CKI γ 2 is also capable of binding to smads *in vitro* and that this interaction is mediated by the very C-terminal region of the smad proteins. Since CKI γ 2 is also a serine/threonine kinase, we examined *in vitro* phosphorylation of Smads and receptors by CKI γ 2 and found that all smads (Smads 1, 2, 3, 4 and 5) and the type II receptor are phosphorylated by CKI γ 2. Additionally, using reporter assays, it was observed that CKI γ 2 dramatically inhibits TGF- β stimulated transcriptional reporter activity. The inhibition of TGF- β mediated transcription is most likely the result of the ability of CKI γ 2 to significantly reverse the transcriptional activity of smad3. Finally, by transiently reducing the protein level of CKI γ 2 it was found that this kinase is critically important for maintaining tight regulation of the TGF- β signaling pathway. The loss of this protein results in a dramatic increase in ligand stimulated transcriptional reporter activity in TGF- β responsive cells.

The fact that the CKI family appears to play a role in controlling both the basal and ligand stimulated activity of the TGF- β pathway implies that this family of kinases may act as an important signal regulator. The significance of these observations remains to be determined, however if the CKI family does ultimately prove to be a necessary regulator, then this would be the first evidence that the CKI family might be involved in the development and/or progression

of cancers in which there is a loss of regulation of the TGF- β pathway. These results taken together demonstrate that the CKI family interacts with several components of the TGF- β pathway and plays a significant regulatory role in the presence and absence of ligand. These observations provide intriguing insight into the maintenance of a major signal transduction pathway involved in the development and progression of many different types of cancers, including breast cancer.

Importance and Implications

The ongoing advances in the understanding and treatment of cancer depend almost unconditionally on the knowledge gained through basic scientific research conducted everyday by countless labs around the world. The increased understanding of how signal transduction pathways work and how mutations in these pathways can ultimately result in uncontrolled cell growth is invaluable to our ability to identify targets for the development of new drugs and improved treatments. The research described above is merely another cog in the wheel of our understanding of the TGF- β signaling pathway. Taken independently this research may seem trivial and insignificant, but when combined with the vast knowledge that we have already accumulated it becomes much more important as we try to determine how this signaling pathway functions and where mutations within this pathway may prove to be the most damaging with respect to regulation. Whether the casein kinase I family ultimately proves to be a major player in the regulation of the TGF- β pathway, or just another minor effector remains to be determined. Regardless of the importance of the casein kinase I family within the framework of the TGF- β signaling pathway, it is becoming more and more apparent that this family of serine/threonine kinases has some significant role to play in the maintenance and regulation of many important

and potentially oncogenic signal transduction pathways. This observation alone, irrespective of the role CKI may have in the TGF- β pathway, may someday make the CKI family members an important target in treating patients with cancer.

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Appendices

Figure Legends

Figure 1: CKI Isoform Expression Pattern in TGF- β Responsive Cells. RNA was isolated from HepG2 cells and HaCaT cells and used for RT-PCR using specific primers for each of the reported CKI isoforms α , $\gamma 1$, $\gamma 2$, $\gamma 3$, δ , and ϵ) and GAPDH as a positive control. This gel is a representative example the expression pattern of both cell lines.

Figure 2: CKI family members interact with smads *in vitro*. Radiolabeled CKI α , CKI δ , and CKI ϵ were individually incubated with GST purified smads bound to glutathione conjugated to sepharose beads, washed with binding buffer, run on a SDS-PAGE gel, dried and exposed to film to visualize.

Figure 3: CKI $\gamma 2$ - ΔN interacts with smads *in vitro*. Radiolabeled smad proteins were incubated with GST-purified CKI $\gamma 2$ - ΔN bound to glutathione conjugated to sepharose beads, washed with binding buffer, run on a SDS-PAGE gel, dried and exposed to film to visualize.

Figure 4: CKI $\gamma 2$ binds to the C-terminal region of smads *in vitro*. Radiolabeled full-length smad proteins or smad proteins with approximately the last 30 amino acids deleted from the C-terminus (smad- ΔC) were incubated with GST purified CKI $\gamma 2$ - ΔN bound to glutathione conjugated sepharose beads, washed with binding buffer, run on a SDS-PAGE gel, dried and exposed to film to visualize. This figure shows Smad1 binding to GST-CKI $\gamma 2$ - ΔN and is representative of the binding pattern of the other smad proteins that were evaluated (i.e. smad2, smad3, and smad4)

Figure 5: The C-terminal tail of CKI ϵ is not required for *in vitro* binding with smads. Radiolabeled full-length smad proteins were incubated with GST purified CKI ϵ C-terminal truncation mutants bound to glutathione sepharose beads, washed with binding buffer, run on a SDS-PAGE gel, dried and exposed to film to visualize. The CKI ϵ C-terminal truncation mutants were made by introducing premature stop codons at the amino acid residues indicated in the schematic.

Figure 6: CKI ϵ interacts with TGF- β receptors *in vitro*. Radiolabeled CKI ϵ was incubated with GST purified TGF- β type I and type II receptors, as well as, deletion constructs of the type II receptor bound to glutathione conjugated to sepharose beads, washed with binding buffer, run on a SDS-PAGE gel, dried and exposed to film to visualize.

Figure 7: CKI ϵ binds to smads and TGF- β type I and type II receptors *in vivo*. Wild type HaCaT (-) cells and HaCaT cells stably expressing CKI ϵ (C) were used to make whole cell lysates. The lysates were then incubated with antibodies to TGF- β type I receptor (TRI), TGF- β type II receptor (TRII), smads2/3 (S2/3), and smads1/5 (S1/5). The antibodies were then precipitated using a 50/50 mixture of ProteinG and ProteinA conjugated to sepharose beads. The immunoprecipitated complexes were then washed, run on a SDS-PAGE gel, transferred to PVDF

membrane, and blotted for CKI ϵ .

Figure 8: CKI ϵ interaction with Smad2/3 *in vivo* is transiently disrupted by TGF- β treatment. HaCaT cells stably expressing CKI ϵ were used to make whole cell lysates. The lysates were then incubated with an antibody to smads2/3. The antibody was then precipitated using a 50/50 mixture of ProteinG and ProteinA conjugated to sepharose beads. The immunoprecipitated complexes were washed, run on a SDS-PAGE gel, transferred to PVDF membrane, and blotted for CKI ϵ .

Figure 9: CKI ϵ interaction with the TGF- β type II receptor *in vivo* is independent of TGF- β treatment. HaCaT cells stably expressing CKI ϵ were used to make whole cell lysates. The lysates were then incubated with an antibody to the TGF- β type II receptor (TBR2). The antibody was then precipitated using a 50/50 mixture of ProteinG and ProteinA conjugated to sepharose beads. The immunoprecipitated complexes were washed, run on a SDS-PAGE gel, transferred to PVDF membrane, and blotted for CKI ϵ .

Figure 10: CKI ϵ phosphorylates smads and the TGF- β type II receptor *in vitro*. Smad proteins and the cytoplasmic domains of the TGF- β type I and type II receptors were fused to GST, purified using glutathione conjugated to sepharose beads and eluted from the beads using free glutathione. These purified proteins were then incubated with purified CKI ϵ in the presence of ATP-P³² for 30 minutes. The reactions were terminated and run on a SDS-PAGE gel, dried and exposed to film to visualize.

Figure 11: CKI γ 2- Δ C phosphorylates smads and the type II receptor *in vitro*. Smad proteins and the cytoplasmic domains of the TGF- β type I and type II receptors were fused to GST, purified using glutathione conjugated to sepharose beads and eluted from the beads using free glutathione. These purified proteins were then incubated with immunoprecipitated Flag-tagged CKI γ 2- Δ C in the presence of ATP-P³² for 30 minutes. The C-terminal deletion construct of CKI γ 2 was made by introducing a premature stop codon into the tail region of full-length Flag-tagged CKI γ 2 in order to reduce autophosphorylation and autoinhibition. The reactions were terminated and run on a SDS-PAGE gel, dried and exposed to film to visualize.

Figure 12: CKI ϵ acts to fine tune SBE-Lux responsiveness to TGF- β . HepG2 cells were transiently transfected with the reporter construct SBE-Lux, and increasing concentrations of either wild type CKI ϵ or the kinase dead version (KD). Cells were treated with TGF- β overnight following transfection and then harvested and assayed for luciferase activity. Transfection efficiency was corrected using β -galactosidase as an internal control.

Figure 13: CKI ϵ enhancement of 3TP-Lux responsiveness to TGF- β requires smad3 but not smad2. HepG2 cell were transiently transfected with the reporter construct 3TP-Lux, and CKI ϵ alone, smad3 alone, smad2 alone, smad3 and CKI ϵ together, or smad2 and CKI ϵ together. Cells were treated with TGF- β overnight following transfection and then harvested and assayed for luciferase activity. Transfection efficiency was corrected using β -galactosidase as an internal control.

Figure 14: CKI ϵ enhances smad3 activation of the SBE-Lux reporter. HepG2 cells were transiently transfected with the reporter construct SBE-Lux, and CKI ϵ alone, smad3 alone, or smad3 and CKI ϵ together. Cells were treated with TGF- β overnight following transfection and then harvested and assayed for luciferase activity. Differences in transfection efficiency were corrected using β -galactosidase as an internal control.

Figure 15: CKI γ 2 represses 3TP-Lux responsiveness to TGF- β . HepG2 cells were transiently transfected with the reporter construct 3TP-Lux, and CKI γ 2 alone, smad3 and smad4 together, or smad3, smad4, and CKI γ 2 together. Cells were treated with TGF- β overnight following transfection and then harvested and assayed for luciferase activity. Differences in transfection efficiency were corrected using β -galactosidase as an internal control.

Figure 16: Transient knock-down of CKI α , CKI δ , and CKI ϵ leads to increased reporter activity in response to TGF- β . HepG2 cells were serially transfected three times with duplexed siRNA oligos designed against either CKI α alone, CKI ϵ alone, or CKI δ and CKI ϵ together. Following siRNA knock-down, cells were transiently transfected with either the SBE-Lux or the 3TP-Lux reporter construct, treated with TGF- β overnight, and then harvested and assayed for luciferase activity. Differences in transfection were corrected using β -galactosidase as an internal control. In order to determine the efficiency of each siRNA duplex to knock-down its target protein, the unused lysates were analyzed by western blot.

Figure 17: Transient knock-down of CKI γ 2 leads to increased transcriptional reporter activity in response to TGF- β . HepG2 cells were serially transfected three times with one of three pSuper vectors containing sequences corresponding to different regions CKI γ 2. Following RNAi knock-down, cells were transiently transfected with either the SBE-Lux or the 3TP-Lux reporter construct, treated with TGF- β overnight, and then harvested and assayed for luciferase activity. Differences in transfection were corrected using β -galactosidase as an internal control. In order to determine the efficiency of each pSuper construct to knock-down CKI γ 2, the unused lysates were subjected to SDS-PAGE, transferred to PVDF membrane and probed with a rabbit polyclonal antibody against CKI γ 2.

Bibliography for Final Report

Manuscripts (in preparation):

Casein Kinase I ϵ Plays a Functional Role in the Transforming Growth Factor- β Signaling Pathway. David S. Waddell, Nicole T. Liberati, Ph.D., and Xiao-Fan Wang. *Duke University Medical Center, Department of Pharmacology and Cancer Biology, Durham, NC 27710.* To be submitted to Journal of Biological Chemistry.

Casein Kinase I γ 2 Negatively Regulates the Transforming Growth Factor- β Signaling Pathway by targeting Smad 3 for Degradation. David S. Waddell, Guo Xing, Nicole T. Liberati, and Xiao-Fan Wang. . *Duke University Medical Center, Department of Pharmacology and Cancer Biology, Durham, NC 27710.* To be submitted to Journal of Biological Chemistry.

Meeting Abstracts:

Casein Kinase I ϵ Regulates the TGF- β Pathway and Provides a Link for TGF- β Activation of the Wnt Pathway. David S. Waddell, Nicole T. Liberati, Jeremy N. Rich, and Xiao-Fan Wang. *Duke University Medical Center, Department of Pharmacology and Cancer Biology, Durham, NC 27710.* Submitted for the April 2002 AACR Meeting.

Casein Kinase I ϵ Plays a Functional Role in the Transforming Growth Factor- β Signaling Pathway. David S. Waddell, Nicole T. Liberati, Ph.D., and Xiao-Fan Wang, Ph.D. *Duke University Medical Center, Department of Pharmacology and Cancer Biology, Durham, NC 27710.* Submitted for the September 2002 Era of Hope Meeting.

Personnel Receiving Pay:

Mrs. Xuefang Bai, (April 2000-April 2001) the original recipient of this award, was funded for one year prior to graduating with a Master of Science Degree in Molecular Cancer Biology.

David Waddell, (April 2001-April 2003) the recipient of a transfer and revised statement of work for this award, was funded for the remaining two years and will graduate with a Ph.D. in Molecular Cancer Biology in the Spring of 2004.

Guo Xing, a third year graduate student who has participated in the research performed on the CKI γ 2 isoform.

Figure 1: CKI Isoform Expression Pattern in TGF- β Responsive Cells

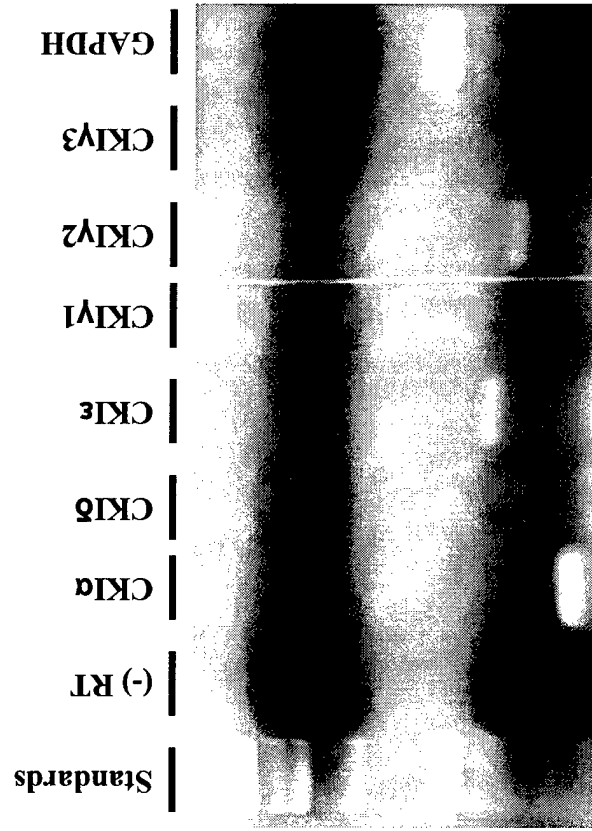


Figure 2: CKI Family Members Interact with Smads *in vitro*

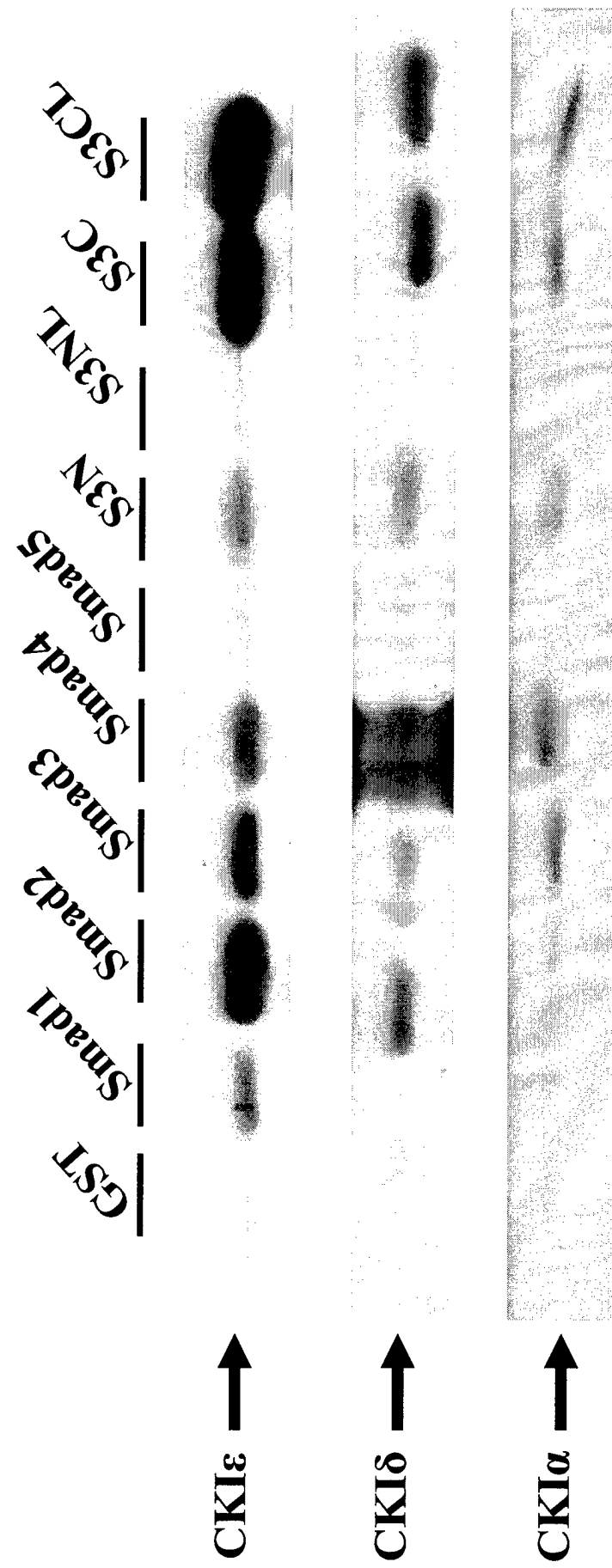


Figure 3: CKI γ 2- Δ N Interacts With Smads *in vitro*

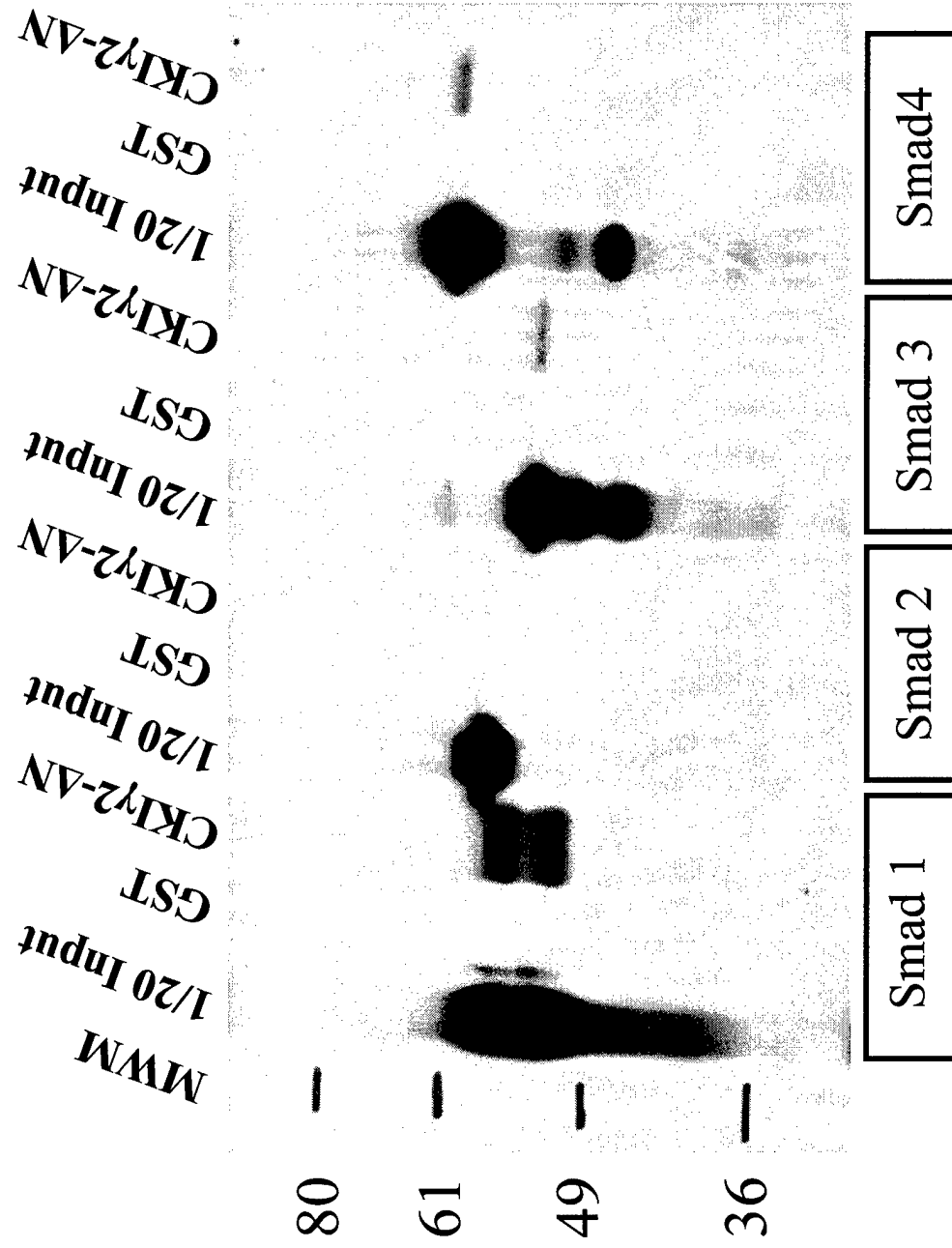


Figure 4: CKI γ 2 Binds to the C-Terminal Region of Smads
in vitro

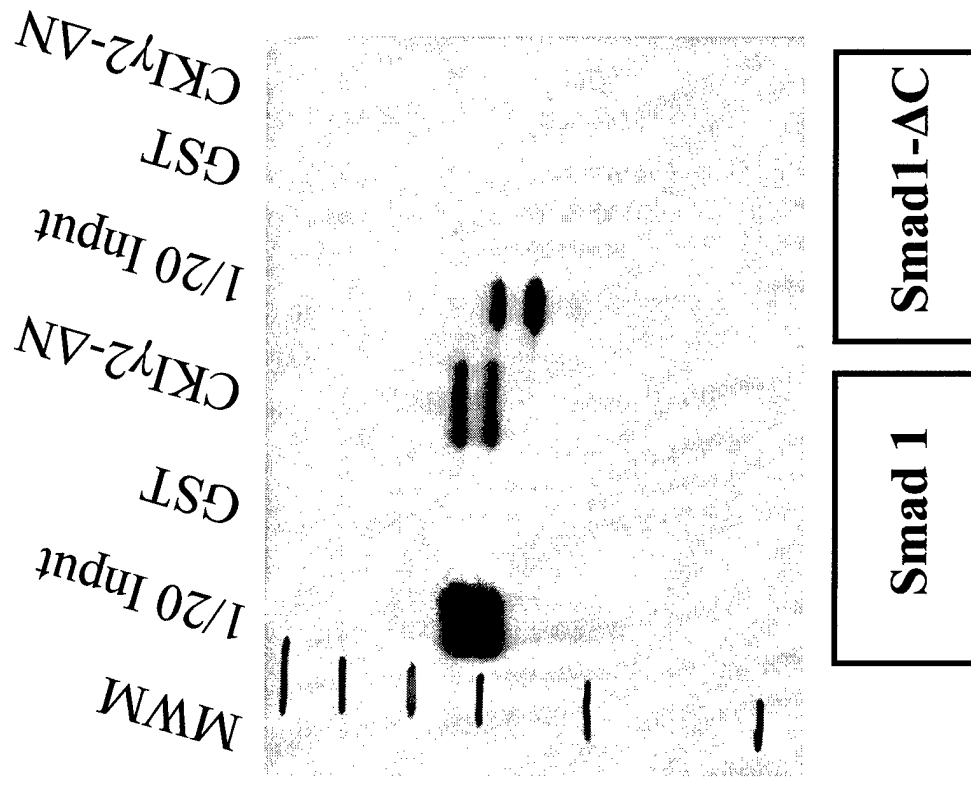


Figure 5: The C-terminal Tail of CKIε is not Required for *in vitro* Binding with Smads

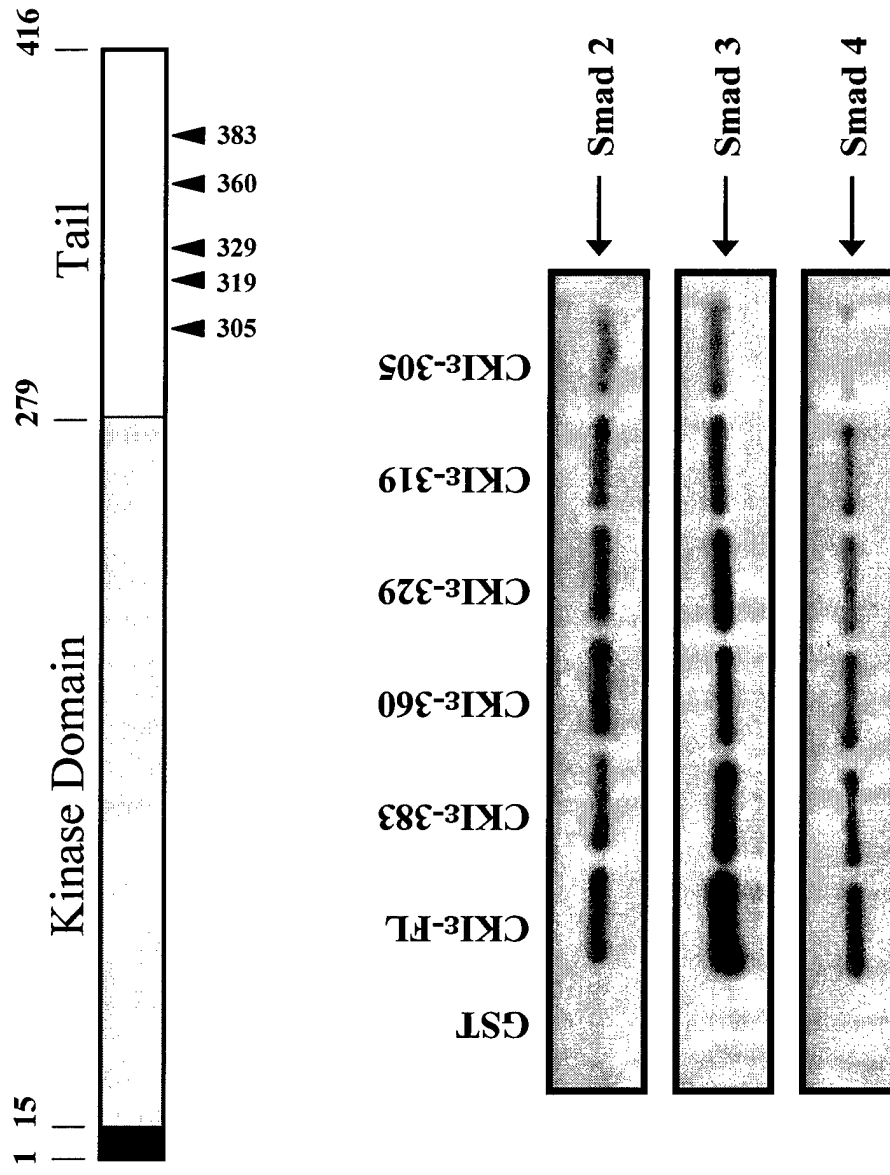
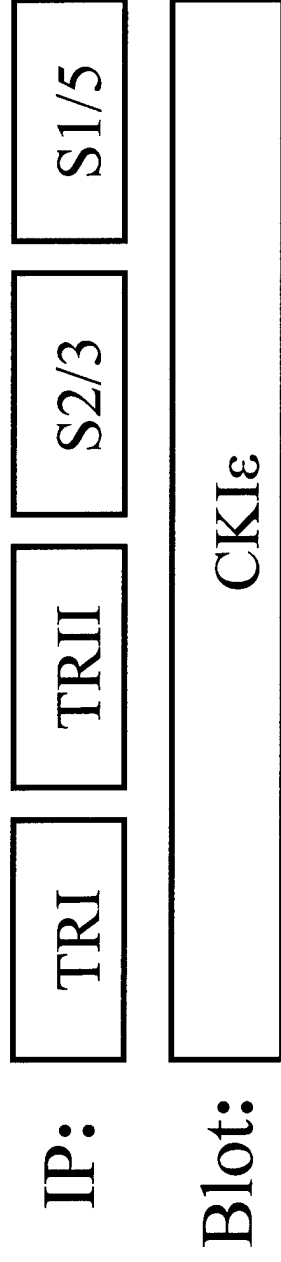


Figure 6: CKI ϵ Interacts with TGF- β Receptors *in vitro*



Figure 7: CKI ϵ Binds to Smads and TGF- β Type I and Type II Receptors In Vivo



M (-) C Con Ab (-) C Ab (-) C Ab (-) C

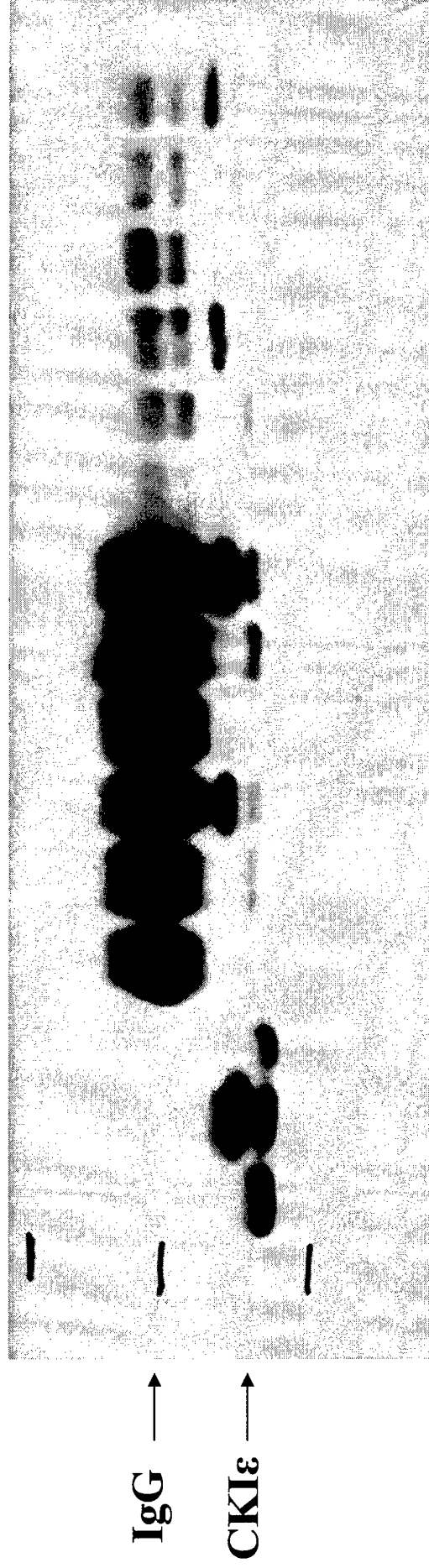


Figure 8: CKIε Interacts with Smad2/3 *in vivo*

Figure 9: CKI ϵ Interacts with TGF- β Type II Receptor *in vivo*

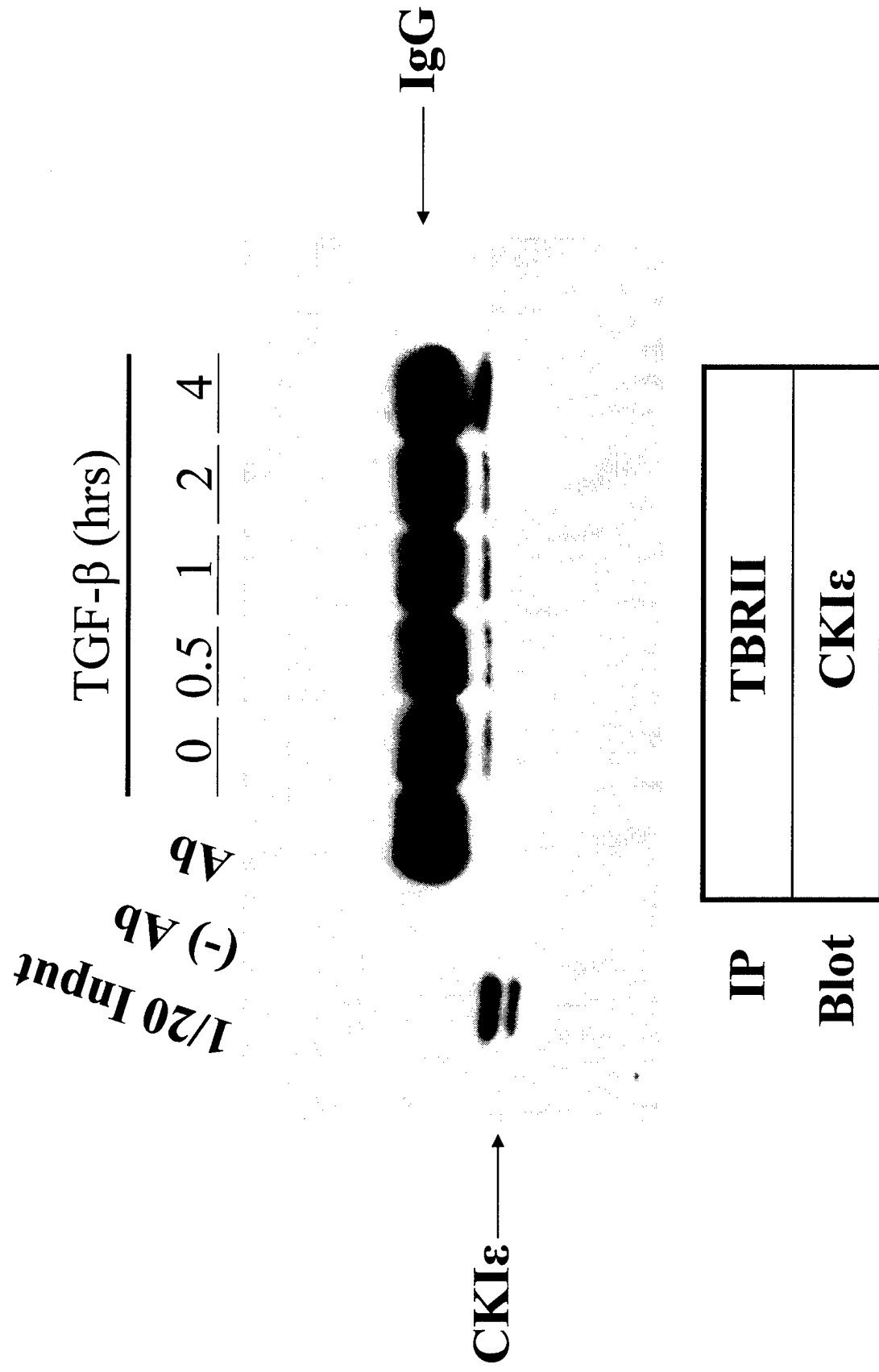


Figure 10: CKIε Phosphorylates Smads and TGF-β Type II Receptor In Vitro

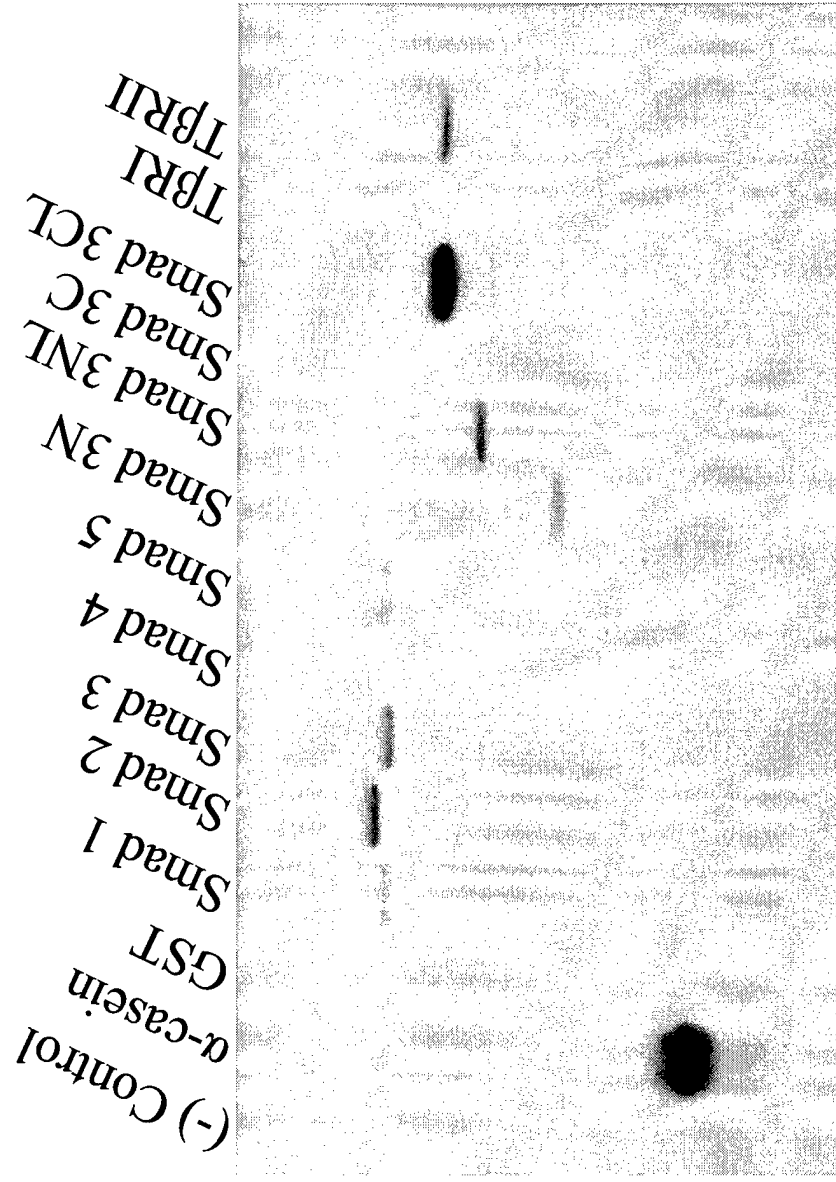


Figure 11: CKI γ 2 Δ C phosphorylates Smad and the Type II receptor *in vitro*

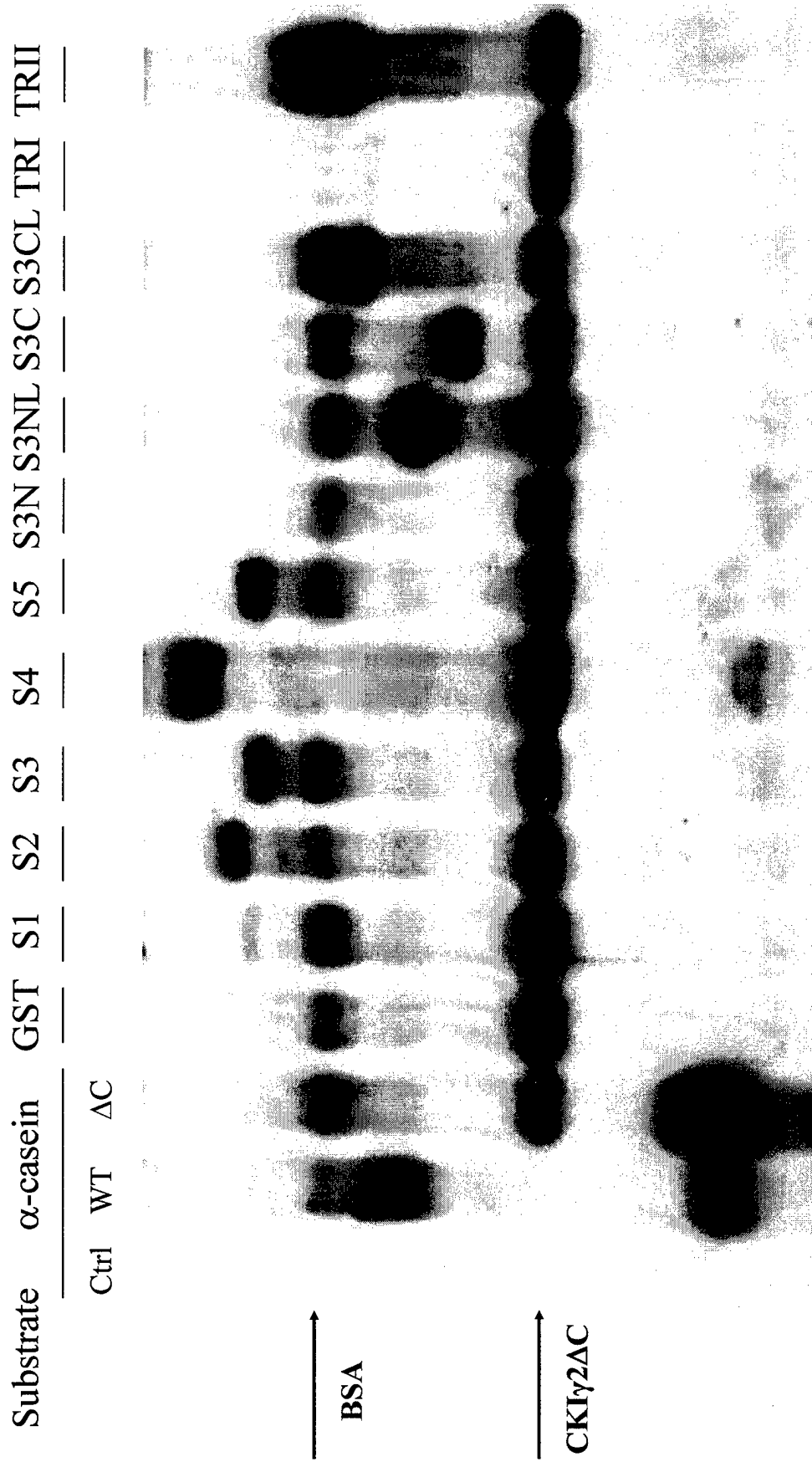
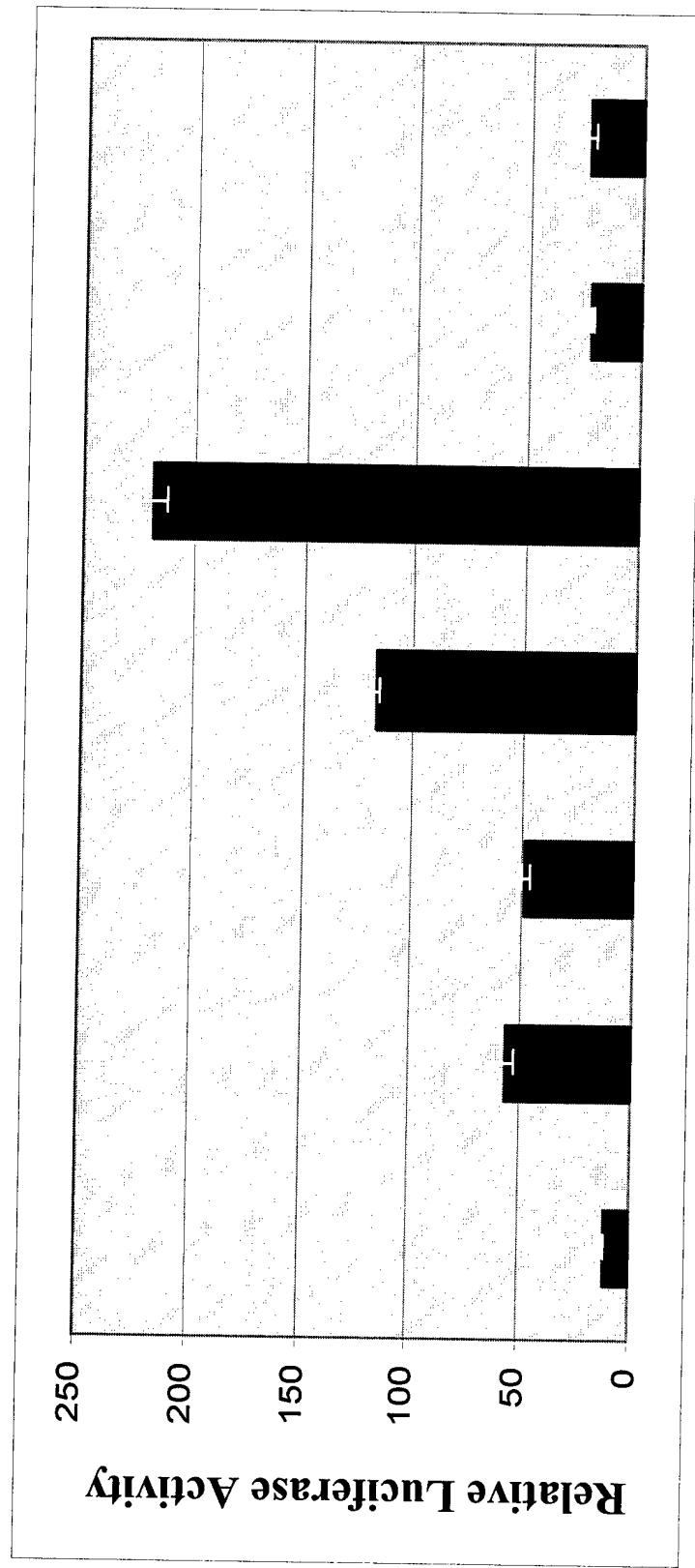


Figure 12: CKIε Acts To Fine Tune SBE-Lux Responsiveness to TGF-β

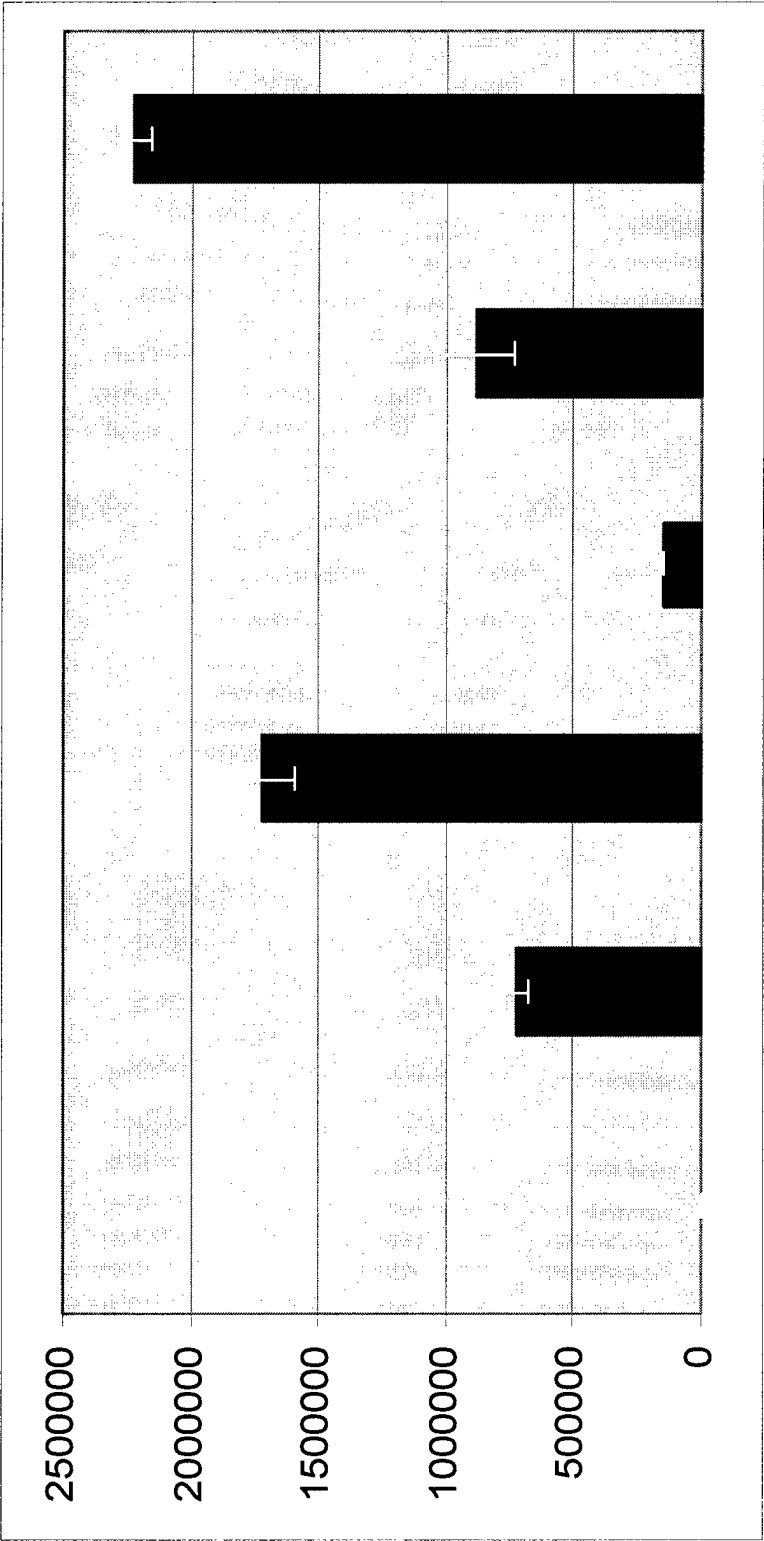


Figure 13: CKI ϵ Enhancement of 3TP-Lux Responsiveness to TGF- β Requires Smad3 but not Smad2



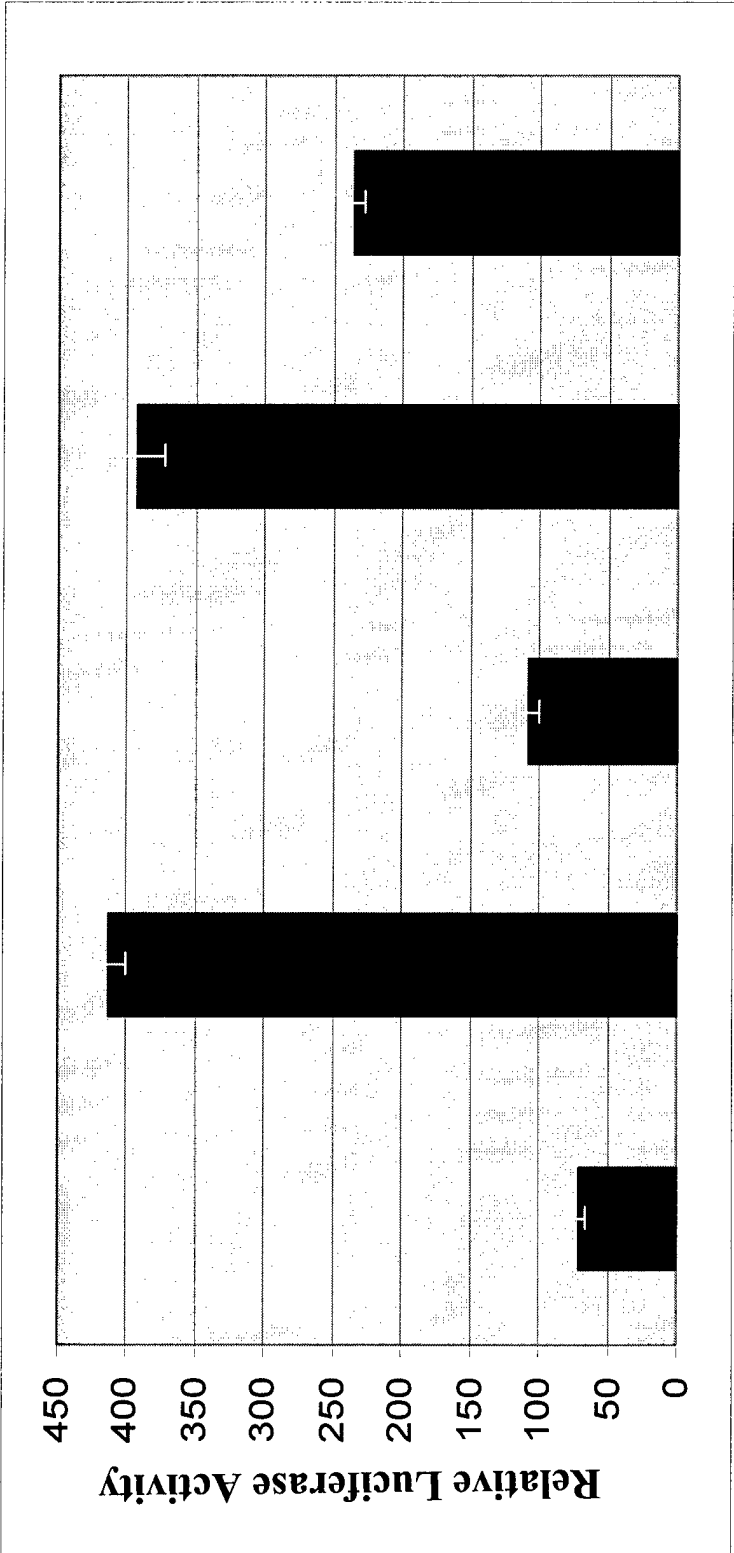
CKI ϵ	-	+	-	+	-	+
Smad3	-	-	+	-	-	-
Smad2	-	-	-	+	-	+
TGF- β	-	-	+	-	-	-

Figure 14: CKIe Enhances Smad3 Activation of the SBE-Lux Reporter



Smad3	-	+	-	+
CKIε	-	+	-	+
TGF-β	-	-	+	+

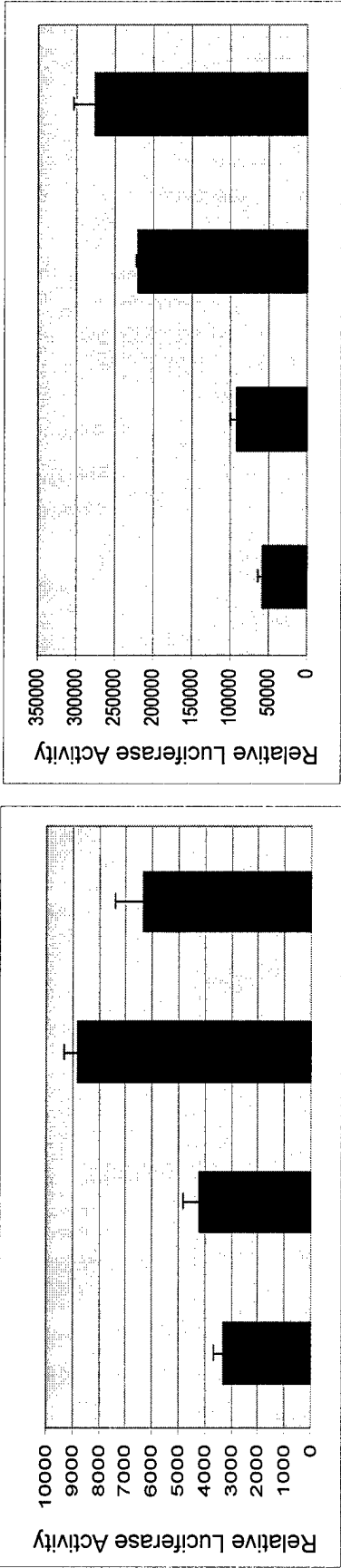
Figure 15: CKI γ 2 Represses 3TP-Lux Responsiveness To TGF- β



Smad 3	-		+
Smad 4	-		+
CKI γ 2	-	+	+
TGF- β	-	+	

Figure 16: Transient Knock-Down of CKI α , CKI δ , and CKI ϵ Leads to Increased Reporter Activity in Response to TGF- β

a) SBE-Lux Luciferase Assay



RNAi ϵ	-	+	-	+	+	-
RNAi δ/ϵ	-	+	-	+	+	-
RNAi α	-	-	+	-	+	+
TGF- β	-	-	-	+	+	+

b) CKI α , CKI ϵ , CKI δ Protein Levels

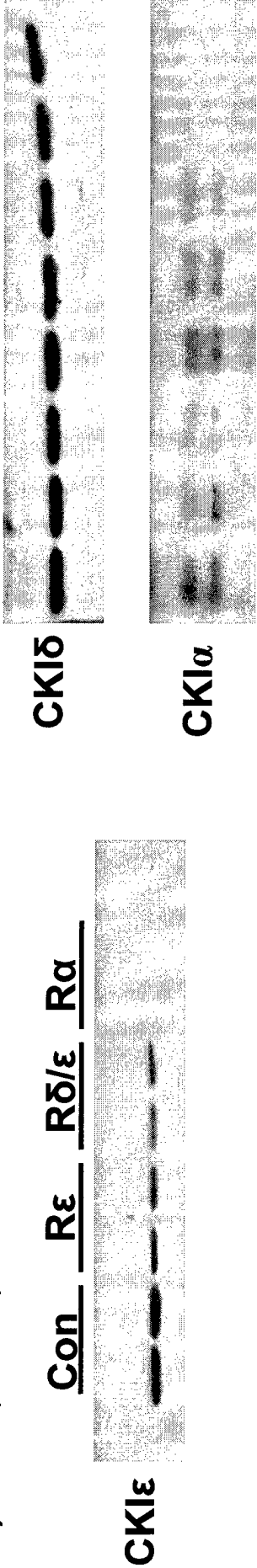
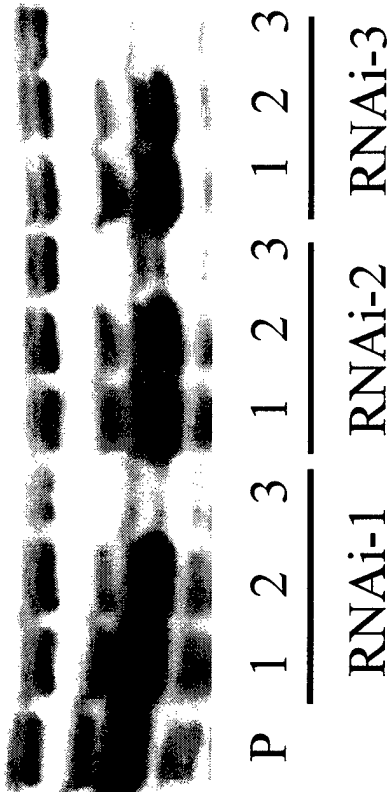
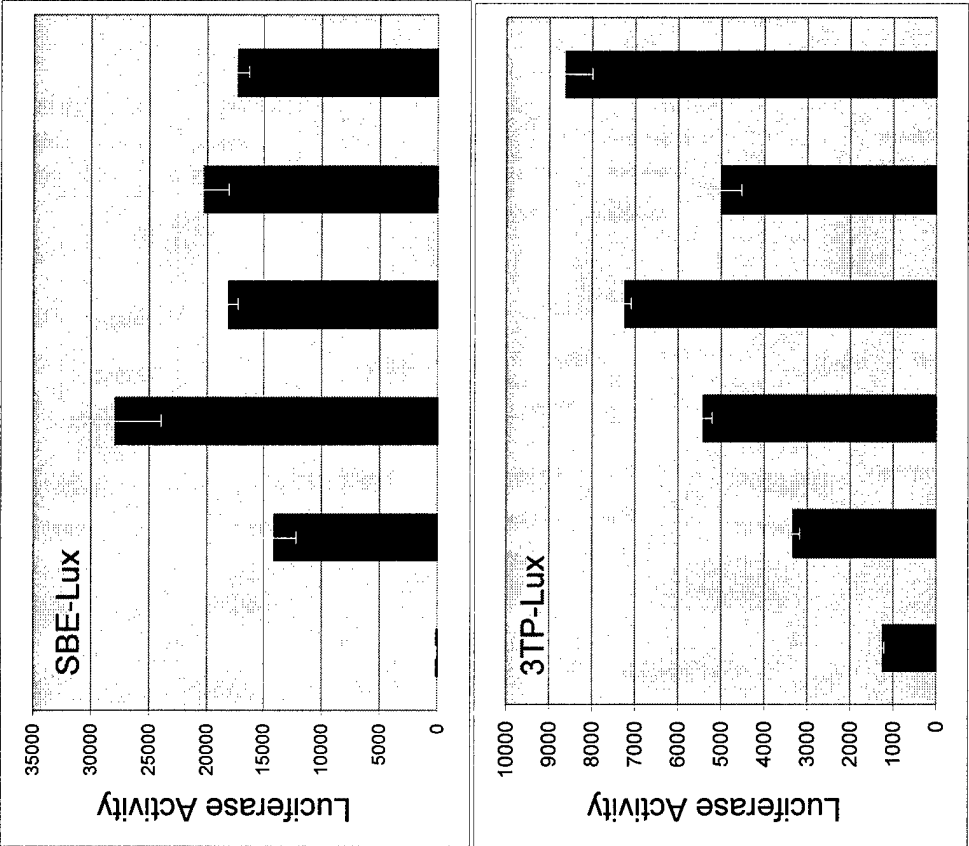


Figure 17: Transient Knockdown of CKI γ 2 Leads to Increased Transcriptional Reporter Activity In Response to TGF- β



RNAi-1	-	+	-	+
RNAi-2	-	+	-	+
RNAi-3	-	-	+	+
TGF- β	-	+	+	+